PEWARNAAN α-ACTININ SEBAGAI METODE PENGUKURAN LUAS PERMUKAAN SEL MODEL KARDIOMIOBLAS (H9C2) YANG DIINDUKSI DENGAN ANGIOTENSIN II

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ABSTRAK

Pendahuluan: Hipertrofi kardiomioblas sebagai karakteristik gagal jantung dapat direplikasi secara *in vitro* menggunakan sel H9C2 yang diinduksi dengan Angiotensin II (Ang-II). Namun pengukuran luas sel sulit dilakukan apabila tanpa pewarnaan khusus. Penelitian ini dilakukan untuk menilai luas permukaan sel dengan dan tanpa pewarnaan α-Actinin pada sel model kardiomioblas H9C2 yang diinduksi dengan Ang II.
Metode: Sel H9C2 ditumbuhkan pada media Dulbecco's Modified Eagle Medium (DMEM) dan disubkulturasi setiap 2-3 hari sampai kepadatan populasi sel 80% sebelum dipindahkan ke T-25 *flasks*. Sel kemudian diinkubasi dengan 600nM Ang II selama 24 jam. Pewarnaan α-actinin dilakukan dengan 4% paraformaldehyde, 0.5% Triton X-100, antibodi primer dan sekunder . Kultur yang telah diwarna kemudian diobservasi menggunakan mikroskop dan diukur luas permukaan selnya menggunakan Image-J. Hasil dianalisa dengan uji *T-test* dan $p < 0.05$ dianggap signifikan.
Hasil: Sel H9C2 tanpa pewarnaan α-actinin tidak menunjukkan perbedaan signifikan pada penghitungan luas permukaan sel pasca induksi Ang-II dibanding kontrol ($3x10^{-9}\mu m \pm$ SD). Pewarnaan α-actinin membantu visualisasi sel H9C2 oleh adanya fluoresensi hijau sehingga memperbaiki akurasi pengukuran luas permukaan sel H9C2 yang diinduksi dengan Ang II. Hal ini ditunjukkan oleh perbedaan yang signifikan antara kontrol vs induksi Ang II ($4x10^{-9}\mu m \pm$ SD vs $9x10^{-9}\mu m \pm$ SD) pasca pewarnaan α-actinin.
Kesimpulan: Pemberian angiotensin II dapat menyebabkan kondisi hipertrofik kardiomioblas dan pewarnaan α-actinin dapat membantu akurasi evaluasi pengukuran luas sel model kardiomioblas (H9C2).

Kata Kunci: *Cardiomyoblast hypertrophy; H9C2; Angiotensin II; α-Actinin Staining.*

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α-ACTININ STAINING FOR MEASUREMENT OF CELL SURFACE AREA IN H9C2 CELLS INDUCED WITH ANGIOTENSIN II

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ABSTRACT

Introduction: Cardiomyoblast hypertrophy as a characteristic of heart failure can be replicated in *in vitro* studies using H9C2 cells induced with Angiotensin II (Ang II). However, Measurement of cell area is difficult to do without particular staining. The study was conducted to assess the surface area of cells with and without α-Actinin staining in H9C2 cardiomyoblast model cells induced with Ang II.
Method: The H9C2 cells grown in Dulbecco's Modified Eagle Medium (DMEM) are passaged every 2-3 days when the cell population density reaches 80% before being transferred to T-25 *flasks*. The cell is then incubated with 600 nM Ang II for 24 hours. α-Actinin staining was performed with 4% paraformaldehyde, 0.5% Triton X-100, primary and secondary antibodies. The cells then observed using a microscope and measurement of cell surface area quantitated using Image-J software. Results analyzed with the *T-test* and $p < 0.05$ are considered significant.
Results: There is no significant difference between H9C2 cells exposed with Ang-II compared to control ($3x10^{-9}\mu m \pm$ SD) without α-actinin staining. α-Actinin staining improved visualization characterized by the presence of green fluorescence thus improving the accuracy of measurement of the surface area of H9C2 cells induced with Ang II. This is indicated by a significant difference between control vs Ang II induction ($4x10^{-9}\mu m \pm$ SD vs $9x10^{-9}\mu m \pm$ SD) post α-actinin staining.
Conclusion: Exposure of Ang II can cause hypertrophic conditions and α-actinin staining improved the accuracy of evaluation in measurement of cardiomyoblast model cells (H9C2).

Keywords: *Cardiomyoblast hypertrophy; H9C2; Angiotensin II; α-Actinin Staining.*

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INTRODUCTION

Heart failure is one of the many diseases that still cause high mortality rates in Indonesia, and even around the world (Nichols et al. 2014). The development of heart failure is originally manifested as cardiac remodeling, a result of cardiac hypertrophy. Cardiac hypertrophy may occur as an adaptive physiological response aimed at improving or at least compensating the heart to function properly (Bernardo & McMullen. 2016).

Cardiac hypertrophy can be mimicked *in vitro* using cardiomyoblast hypertrophy in cell line. H9C2 cells are derived from rat's heart and are commonly used for hypertrophy induction since the cells bear similarities to human cardiomyocyte (Watkins et al. 2011). *In vitro* studies of cardiac hypertrophy have been widely used to investigate the pathogenesis and mechanisms of heart failure, which can be induced by various stimuli such as phenylephrine (Dong et al. 2018) or angiotensin II (Ang II) (Chiang et al. 2018).

Ang II is a fairly good hypertrophic stimuli and can mimic cardiac hypertrophy induced by high blood pressure in hypertensive conditions (Ying et al. 2014). The hypertrophy in cardiomyoblast cells can be assessed by looking at the increase in the size of cardiomyoblast cells surface area. There are many methods to assess cardiomyoblast surface area such as α-actinin (Jeong et al. 2015) and crystal violet (Jamhiri et al. 2019) stainings. However, such methods have not been widely used in Ang II induction for hypertrophy in H9C2 cells. In this report, hypertrophy induction by Ang II and the measurement of the cell surface area using actin filament staining by α-actinin are described step-by-step.

MATERIALS AND METHODS

Preparation of reagents and cell culture medium

This *in vitro* study used the H9C2 embryonic rat heart-derived cell line (American Type Culture Collection, ATCC) which is cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L of glucose, 1.5 g/L sodium bicarbonate and 110 mg/L sodium pyruvate, then given supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), penicillin (100 unit/ml) and streptomysin (100 mg/ml) in a petri dish. The whole process of reagent preparation and cell culture medium is carried out in a laminar air flow (LAF) cabinet.

Cell culture treatment

The H9C2 cells were subsequently incubated in a humid incubator (5% CO2/95% O2) at 37°C. The cells were passaged every 2–3 days when the cell population density reached 80% confluency. the incubated H9C2 cells were then transferred to T-25 flask and exposed to Ang II (600 nM) and incubated for 24 hours at 37°C in 5% CO2/95% O2. The concentration of Ang II was chosen based on our previous pilot study (unpublished data). A group of cells that were not exposed to Ang II acted as the control for comparative purpose.

Measurement of Cell Surface Area

The measurement of cell surface area was modified from the method described by Jeong et al. (2015). After being treated with Ang II, the H9C2 cells were then grown on coverslips and then immunofluorescence staining was performed. Firstly, the H9C2 cells fixed with 4% paraformaldehyde for 10 minutes, permeabilized with 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 10 minutes. Then, the liquid inside the coverslips was removed. FBS (10%) was used to block the cells for an hour at 37°C. The cells were then incubated with primary antibody mouse anti-sarcomeric α-actinin (1:200 dilution; ab9465; Abcam, Cambridge, MA, USA) overnight at 40°C. Then the cardiomyoblasts were incubated with Alexa 488-conjugated anti-mouse secondary antibody (1:200 dilution; Cat. No.: A-11059, Invitrogen, USA) at room temperature for an hour. About 100 cells were observed under a microscope equipped with 40x objective lens and epifluorescence filter (Olympus Optical, Japan) by two blinded assessors. Cell surface areas were measured using NIH Image-J software.

Statistical Analysis

Each experiment was repeated at least three times. Data were presented as a mean and standard deviation value (SD). The data were

analyzed with T-test using SPSS software. p <0.05 was considered statistically significant

RESULT

As seen in figure 1A & B, without α-actinin stain, the H9C2 cells are not clearly visible which hinders cells surface area measurements and increase the risk of bias while measuring the surface cell area. Actinin-α stains greatly improved cell visibility and measurements as seen in figure 1 C and D which made cell visualization, comparison and measurements possible.

There is no significant difference between H9C2 cells exposed with Ang-II and control **(Figure 2)** due to lack of cell visualization and problems occurred during cell surface measurement. Using α- actinin staining, exposure to Ang II at 600 nM is shown to increased cell size area and actin fiber rearrangement (10 cells which are spindle shaped and mono-nucleated from each of the three experiments were taken for measurement) compared to normal control to H9C2 cells, shown by the α-actinin-stained cells, indicated by the green fluorescence (**Figure 1C & D**). cells that retained mitogen activated protein kinase (MAPK) caused the hypertrophic features. Quantitative measurement of the cell surface area showed that Ang II significantly increase the size of the cell surface area compared to the control (**Figure 2**). It was found that the H9C2 cells induced with Ang II had an increasement in the size of cell surface area (average enlargement of 4000 nanometers) compared to the H9C2 cells without Ang II induction.

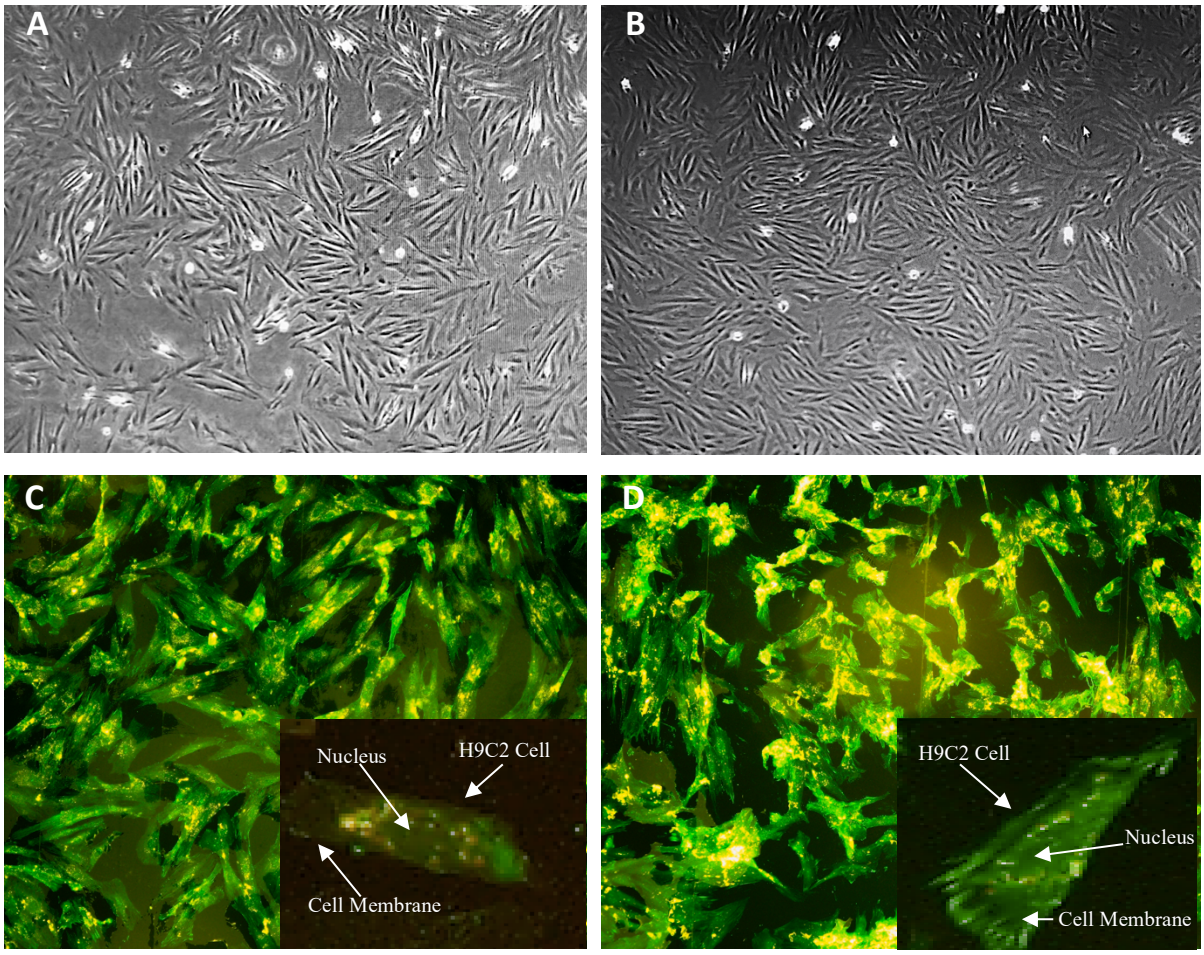

Figure 1. Representative photograph of H9C2 cardiomyoblast without (A and B) and with *α- actinin* stain (C and D) from control (10 cells with no hypertrophic induction) in spindle shape with smaller surface area (C) and exposed to Ang II (10 cells with mono-nucleated) in spindle shape with larger surface area (D) under an immunofluorescence microscope at 400X magnification. Hundreds of cells counted are in the size of square micrometre.

DISCUSSION

As seen in **Figure** 1, Ang II increased cell surface area significantly compared to control at the dose 600 nM ($4x10^{-9}$µm in normal size and $9x10^{-9}$µm in hypertrophy size). This project was a part of a larger project which measures the effect of herbal plant extracts that are often used by certain communities in southeast asia to evaluate the pharmacological potential of the plant parts that are often unused, for method validation purposes (unpublished findings). However, the emphasis in this study is on the α-actinin staining process used.

The H9C2 cells (which are spindle shaped and mono-nucleated) used in this study showed good results and could mimic the condition of cell hypertrophy in humans. Generally, H9C2 cells are spindle to stellate cells, which can be mononucleated or multinucleated (Witek et al. 2016). The H9C2 cells obtained from rats have been widely used in previous studies and showed hypertrophic responses *in vitro* with primary neonatal cardiomyocyte cells as the comparison (Tang et al. 2020; Watkins et al. 2011). The cells were originally derived from rat embryonic ventricular tissues, which are believed to have similarities with cardiomyocyte cells in the ventricles of the human heart (Prathapan et al. 2013).

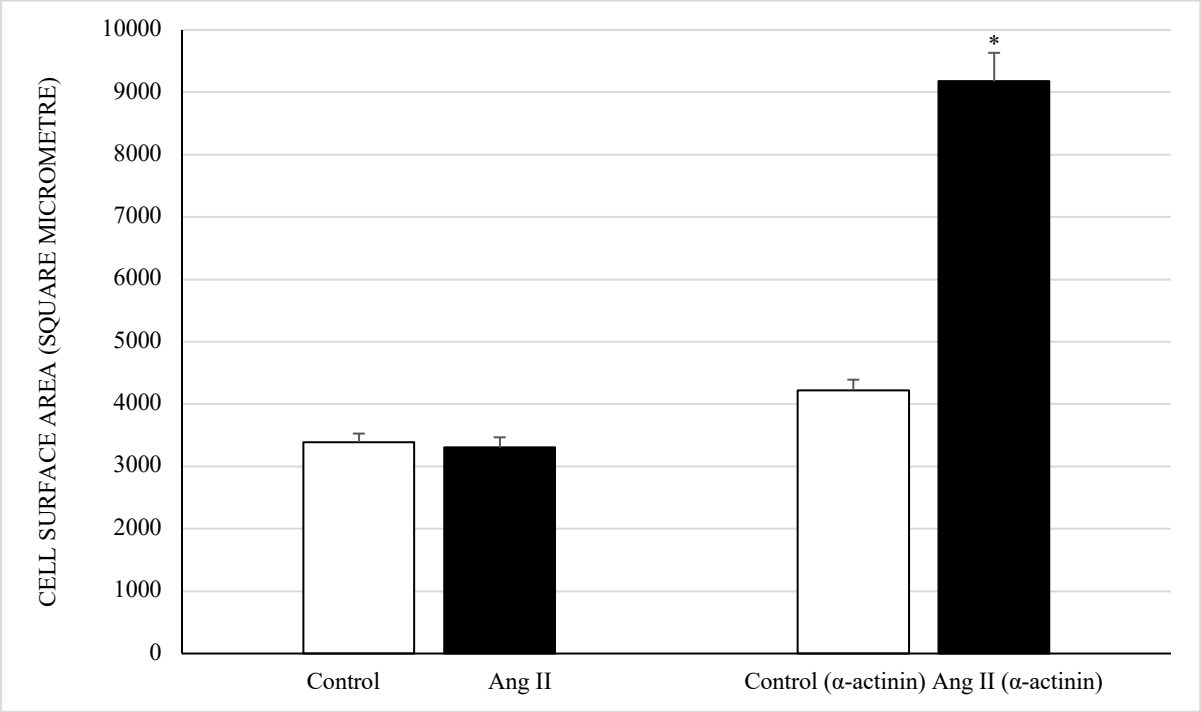

Figure 2. Cell surface area in H9C2 cells that were exposed to angiotensin II (Ang II with α-actinin, 600 nM) relative to control witih α-actinin after 24 hours. The bars represent mean ± SD (n=3).*p<0.05 vs. control.

Ang II has been used to induce cardiomyoblast hypertrophy (Chiang et al. 2018) ($2x10^{-9}$µm-$3x10^{-9}$µm in normal size and $7x10^{-9}$µm-$8x10^{-9}$µm in hypertrophy size) (Watkins et al. 2011). Its hypertrophic property is similarly shown in the current experiment. Ang II stimulates mitogen activated protein kinase (MAPK) signalling cascade through the activation of transforming growth factor- β1 (TGF-β1) in the rat cardiomyoblast following pressure overload condition (Zhang et al. 2000). Previous study declare that TGF-β1 signalling pathway dependent to MAPK in order to merges Ang II with its receptor and induce hypertrophy condition as the result. cells that retained transforming growth factor- β - activated kinase 1 (TAK1) expression showed the classic hypertrophic features in cell size and actin fiber rearrangement in response to Ang II induction (Watkins et al. 2012). Another study stated that the activation of cAMP response element-binding protein (CREB) by Endothelin-1 (ET-1) and Phenylephrine (PE) is also involved in the hypertrophy condition (Subedi et al. 2017).

The cardiomyoblast hypertrophy can be assessed by measuring the cell size with the

number of cells unaffected by the presence of hypertrophic conditions. There are few techniques that can be used like measurement of individual cell images captured by a digital camera (Prataphan et al. 2013). α-actinin staining in this study showed both advantages and disadvantages. The disadvantage of the technique is unclear cell border and could be subject to biasness. To reduce this problem, the F-actin of the cells could be stained using antibodies, like α-actinin. α-Actinin is a cytoskeletal protein, especially in cardiac and skeletal muscle. Its antibody (anti-α-actinin) stains the Z-line of myofibrils in the heart (Morris et al. 2020) and sarcomere in skeletal muscles (Ichinoseki-Sekine et al. 2012). The staining produces clear outline and excellent brightness of the cells that can be viewed under a fluorescence microscope. This enables better quantification, reduces biasness and less variations among the cells. However, α-actinin staining also has few shortcomings. The technique requires the use of primary and secondary antibodies which are quite costly, and a fluorescence microscope. The step-by-step technique described in this article for the measurement of cell size is hoped to facilitate studies that involve hypertrophic features.

CONCLUSION

Exposure of Ang II can cause hypertrophic condition in cardiomyoblast model and the measurement of cardiomyoblast cell size can be employed using α-actinin staining to evaluate cell hypertrophic features by Ang II, with certain advantages and disadvantages.

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